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Fluorescence Lifetime based Corneal Metabolic Imaging

Ana Batista[a], Susana F. Silva[a], José Paulo Domingues[a,b] and António Miguel Morgado[a,b*]

[a] *IBILI – Institute for Biomedical Imaging and Life Sciences, Faculty of Medicine, University of Coimbra, Coimbra, Portugal*
[b] *Department of Physics, Faculty of Sciences and Technology, University of Coimbra, Portugal*
amgbatista@fmed.uc.pt, susana.fig.silva@gmail.com, jpd@uc.pt, miguel@fis.uc.pt

Abstract

We are developing a time-gated fluorescence lifetime imaging microscopy (FLIM) instrument for *in vivo* metabolic imaging of corneal tissues, based on the fluorescence of metabolic co-factor FAD. Here we report on the first results of this project, namely on ex vivo measurements done with a time correlated single photon counting FLIM instrument, and on the first measurements done with the time-gated microscope prototype.
Ex-vivo measurements with rat corneas show that it is possible to image FAD fluorescence from corneal epithelial layer and to obtain information from its fluorescence decay parameters that correlates to their metabolic activity. Measurements with test-targets and fluorescence lifetime standards show that the prototype of the time-gated fluorescence lifetime microscope for *in vivo* FAD imaging has the precision and the accuracy, as well as the timing and lateral spatial resolutions, required for such measurements.

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Keywords: Metabolic imaging; Cornea; Fluorescence lifetime

1. Introduction

Diseases of the cornea, the most anterior structure of the eye, have a large impact on life quality as they may cause repeated episodes of pain, severe visual impairment or even permanent loss of vision. It is known that cell metabolic alterations are the first signs of several corneal pathologies. Therefore, the assessment of these alterations can be of outmost importance to provide the clinician the advantage of diagnosing corneal cells dysfunction prior to its pathologic expression.

* Ana Batista. Tel.: +351 239-480200; fax: +351 239-480217.
amgbatista@fmed.uc.pt

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Metabolic alterations can be assessed noninvasively using the fluorescence emission of metabolic co-factors nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate, namely their reduced forms NADH and NADPH, usually identified mutually by the acronym NAD(P)H, and flavin adenine dinucleotide, in its oxidized form, FAD. It has already been proven that these co-factors can provide information about corneal cells redox state, both using one photon [1;2] and two-photon excitation methods [3;4].

In the case of NADH and FAD, measurement of the fluorescence lifetime allows the discrimination between free and protein-bound components. Both co-factors show a double exponential decay, with a short and a long lifetime component. While for NADH the short component corresponds to the free state, in FAD the short component is due to the protein-bound state [5;6]. Given the mechanism of aerobic respiration, free/bound ratios of the metabolic co-factors are good indicators of the cells metabolic activity. These ratios can be obtained through fluorescence lifetime imaging microscopy (FLIM). FLIM's potential as a clinical optical imaging modality has already been reported on age-related macular degeneration [7], diabetic retinopathy [8], basal cell carcinomas [9], and epithelial cancer [10]. An updated review of cell metabolic assessment based in fluorescence was recently published [11].

Although several advantages can be associated with two-photon excitation, namely inherent axial optical sectioning and deeper tissue penetration, it requires high laser power densities which can be hazardous to the cornea. While safety tests must still be performed to evaluate the use of two-photon excitation systems for *in vivo* ocular imaging, one-photon FLIM systems were already applied for *in vivo* retinal imaging.

Here, we report on our work concerning corneal metabolic imaging based on one-photon FLIM using FAD fluorescence as contrast agent. We are currently developing a fluorescence lifetime microscope for *in vivo* corneal metabolic imaging based on FAD fluorescence. *Ex vivo* feasibility studies were performed with a modified commercial fluorescence lifetime microscope, using rat corneas. The results of these studies allowed us to define the corneal fluorescence lifetime microscope specifications. A first version of this instrument was built and tested for precision, accuracy and resolution.

2. *Ex vivo* FAD imaging

2.1. Methods

The feasibility of recording corneal FAD FLIM images was evaluated in an animal study. Male *Wistar* rats aged 6 weeks were used. The animals were maintained at 22 ± 2ºC, with 12 h light/dark, and free access to water and food. All procedures were approved by the Commission of Ethics of Faculty of Medicine of the University of Coimbra, which follows the Directive 2010/63/EU of the European Parliament and of the Council. The animals were sacrificed, and their eyes removed and placed in Hank's Buffered Salt Solution (HBSS). Corneas were dissected in HBSS at room temperature.

Alterations to FAD fluorescence lifetime with alterations to cell metabolism were determined. The superficial and basal epithelial layers of e*x-vivo* corneas were imaged 0h, 48h and 72h after animal sacrifice (*p1*, *p2* and *p3* time points, respectively). The fellow-matched corneas were used as a control for assessing corneal cells viability.

Fluorescence lifetime images were acquired using the Time-Correlated Single Photon Counting (TCSPC) technique [12], with a PicoQuant MicroTime 100 (PicoQuant GmbH, Berlin, Germany) system coupled to an Olympus BX51 Microscope (Olympus Corporation, Tokyo, Japan). The fluorescence excitation source is a 440 nm pulsed diode laser emitting 80 ps pulses at a frequency of 40 MHz. These pulses are conveyed to the sample by the microscope optics and a 40x air objective, with 0.65 numerical aperture. Fluorescence light is collected by the microscope, filtered by a 490 nm dichroic mirror and a 500 nm long pass filter and detected by a single photon counting photomultiplier (PMA Detector). A dedicated time to digital converter (PicoQuant Time Harp TH200), hosted in a PC computer, measures the time delay between the excitation pulse and the detection of first fluorescence photon. For low illumination, the histogram of the first fluorescence photon arrival times corresponds to the convolution between the sample decay profile and the instrument response function (IRF). Imaging is achieved by scanning the sample with a piezoelectric table. Figure 1a shows the schematic of the instrumentation setup. We modified the instrument optical setup in order to allow the acquisition of reflectance images (Fig. 1b), enabling the identification of the imaged corneal layer through its morphologic characteristics. In the reflectance configuration, the dichroic mirror is replaced by a wideband beam splitter.

The IRF was determined with an Erythrosin B solution in water (τ = 90 ns) for proper reconstruction of the fluorescence decay, through a reconvolution and non-linear least square fit process [13]. Data was processed with SymPhoTime v5.3 Software (PicoQuant GmbH, Berlin, Germany). Goodness of fit was evaluated by the reduced qui-square and by inspection of the residuals plot.

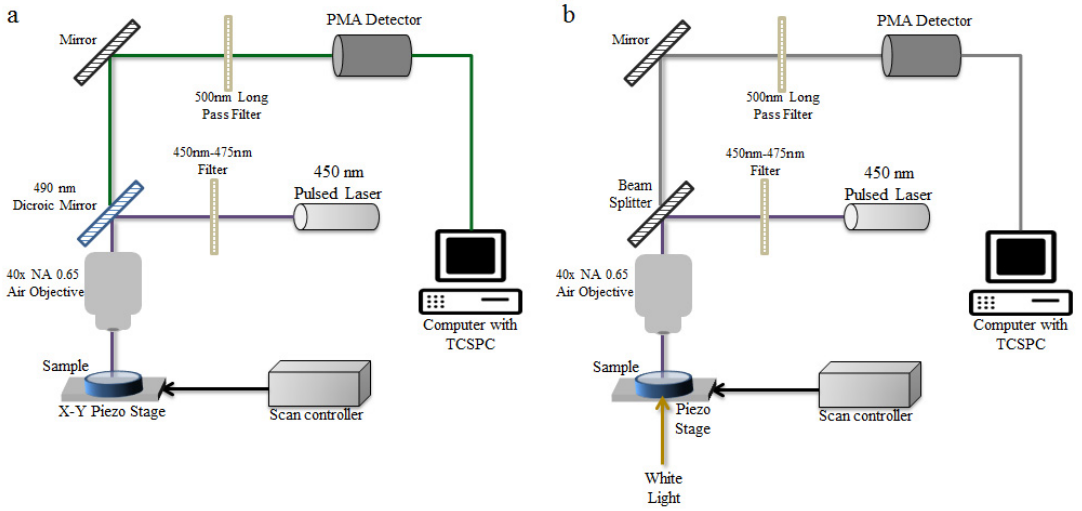

Figure 1 – Optical setup for *ex-vivo* FAD fluorescence lifetime imaging (a) and reflectance imaging (b)

Corneal cells viability in the control eye was assed using the MTT assay, following a protocol adapted from Imbert et al. [14]. This technique is based in the reduction, by viable cells, of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to purple formazan crystals. Corneas were incubated in 200 µl of MTT solution (2 mg/ml) for 2h at 37ºC. After incubation, the sample was rinsed twice with 1 ml of saline solution for 1 min and minced with surgical scissors. The formazan crystals were extracted in 800 µl of Dimethyl sulfoxide (DMSO) on a rotating platform (80 rpm) for 1h. Formazan absorbance was measured at 570 and 620 nm using Synergy HT Multi-mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT, U.S.A.). DMSO was used as blank.

MTT reduction was computed as the difference between the absorbance at 620 nm and 570 nm. Data is presented as percentage of control, with the control being the MTT reduction 0h after animal sacrifice.

2.2. Results

We acquired *ex-vivo* fluorescence lifetime images of superficial and basal epithelial cells of *Wistar* rat corneas (Fig. 2a). The acquired fluorescence lifetime images do not have enough morphologic information to identify the imaged layer. This identification is achieved through reflectance images as the one presented in Figure 2b.

TCSCP data presented a double exponential behavior for all time points. Fluorescence lifetimes and relative contributions to the decay are shown in Table 1.

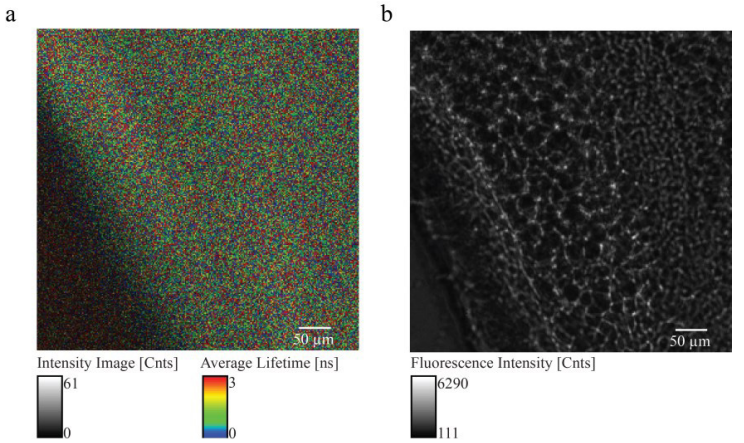

Figure 2 – Fluorescence lifetime image (a) and respective reflectance image (b) of the epithelium of Wistar rat corneas.

Table 1. Fluorescence lifetimes (τ_1 and τ_2) and relative contributions (A_1 and A_2) of *Wistar* rat corneas (mean (SD)).

A_1 [%]	τ_1 [ps]	A_2 [%]	τ_2 [ps]
91.5 (0.6)	138 (20)	8.5 (0.6)	2380 (272)

The measured fluorescence lifetimes are in agreement with values reported in the literature for the metabolic co-factor FAD. The fastest component is likely to represent the protein-bound FAD and the slowest the free component of this co-factor. Nakashima et al. [5] have shown that protein-bound FAD lifetime depends on the conformation of the protein (monomeric or dimeric form), measuring 130 ± 20 ps for the monomeric form and 40 ± 10 ps, when bound to the dimeric form. The authors also report the value 2300 ± 700 ps for free FAD component.

The highest contribution to the decay is associated with protein-bound FAD (over 90%). This is related with high metabolic activity. As mentioned, FAD plays an important role in several steps of the aerobic respiration. For instance, FAD is the primary electron donor and interacts with succinate dehydrogenase (respiratory complex II) in the mitochondrial inner layer. The binding of this co-factor to that mitochondrial complex is essential for an efficient electron transport and energy production [15].

Changes on cell metabolism will cause changes in measured FAD lifetimes and relative contributions. To observe these variations, fluorescence lifetime images of *Wistar* rat corneas were acquired at time points *p1*, *p2* and *p3*.At *p2* and *p3*, we observed a decrease in the relative contribution of protein-bound FAD and an increase in the fluorescence lifetime of this component (Figure 3). These results are consistent with a decrease in metabolic activity [10] due to cell death.

Cell viability was assessed by MTT assay using the fellow cornea, in order to control fluorescence lifetime results. MTT is reduced during the metabolic pathways enabling one to infer on the amount of viable cells present in the sample. A decrease in cell viability, caused by the decrease in tissue metabolic activity, was observed at *p2* and *p3* (Figure 3c). Qualitatively, these results agree with the data from FAD. However, the MTT assay shows a large viability decrease between *p1* and *p2* (approximately 40%). The variation observed with the fluorescence measurements is not so large. While the MTT assay gives information about the cell viability of the whole corneal tissue, fluorescence measurements retrieve only information from epithelial cells viability. This may contribute to the differences observed in the magnitude of viability decrease.

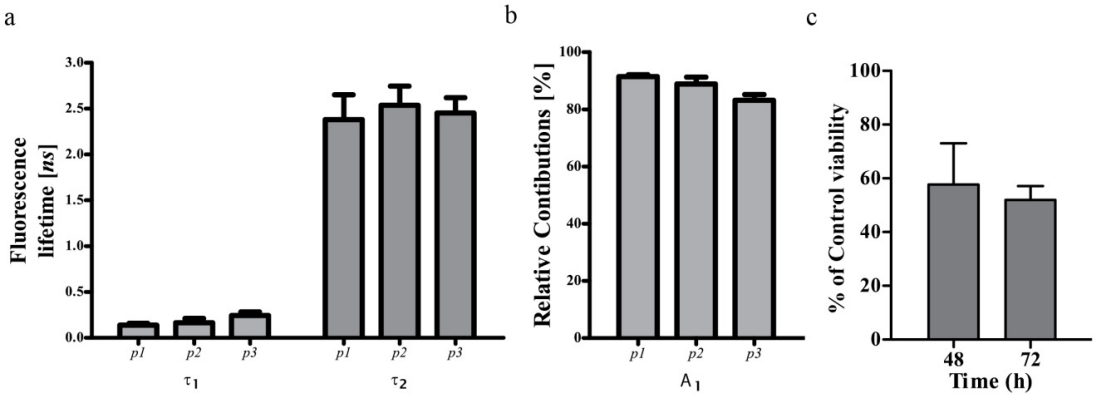

Figure 3 – Metabolic changes assessed by FAD fluorescence lifetimes (a) and relative decay contributions (b) at time instants *p1*, *p2* and *p3*. (c) Corneal cells viability assessed by MTT assay. Values are presented as a percentage of control.

Although the total number of animals used at each time point was small, the results are promising. They show that it is possible to image FAD fluorescence lifetime and to correlate the fluorescence decay parameters with cell viability. This is an indirect measure of cell metabolic activity. A direct correlation requires the acquisition of fluorescence lifetime images in corneas with controlled metabolic alterations, induced by electron transport chain inhibitors. This study is currently ongoing.

The *ex-vivo* measurements show that it is possible to acquire FAD fluorescence lifetime images in rat corneas. However, in vivo requirements for ocular imaging do not seem compatible with an implementation based on the TCSPC technique, due to the long acquisition times required for an adequate signal to noise ratio. A precise recovery of a double exponential decay demands a number of counts per pixel larger than 1000 [16], which will be difficult to obtain with an acquisition time compatible with ophthalmological exam requirements. Therefore, we selected a different technique to be the basis of fluorescence lifetime corneal microscope for *in vivo* measurements.

3. Time-gated wide-field corneal fluorescence lifetime microscope for *in vivo* FAD imaging

3.1. Instrumentation

The corneal fluorescence microscope for *in vivo* FAD metabolic imaging of corneal tissues will be based in the time-gated imaging technique [17]. This is a wide-field technique, with all image pixels being acquired simultaneously. Briefly, a pulsed picosecond diode laser is used to excite the sample and to generate the trigger pulse for an ultrafast gated intensified CCD camera. This camera has an electron multiplier, usually a double microchannel plate (MCP) installed between a photocathode and a phosphor screen. By applying a high-voltage pulse between the photocathode and the MCP, is it possible to vary the camera gain, gating the camera like an ultra-fast electronic shutter. The fluorescence lifetime image can be retrieved by scanning the delay between the excitation and the gating pulses and analysing equivalent pixels from the images recorder for each gating delay.

Currently, we have concluded the first prototype of the corneal fluorescence microscope. The system is based on a 80 ps, 40 MHz pulsed diode laser system composed by a laser head (PicoQuant LDH-P-C-440M) with a central wavelength of 443 nm and a multi-channel picosecond laser driver (PicoQuant PDL 828) controlled by computer through USB and a dedicated software application. The PDL 828 module provides a NIM signal synchronous to each laser pulse. At 40 MHz, the maximum average optical power is 20 mW.

Fluorescence detection and imaging is performed by an ultrafast time-gated intensified CCD camera system (PicoStar HR, LaVision GmbH, Goettingen, Germany). The system includes a High Rate Image Intensifier Control Unit (HRI), a Delay Unit and PC desktop computer with image acquisition and control software (DaVis). The intensified camera has single-photon sensitivity and can be gated at frequencies ranging from 20 to 80 MHz. Gate widths accepted vary between 200 ps and 1000 ps in 100 ps steps. The camera system includes a VGA CCD sensor with 640 x 480 pixels.

The HRI control unit is responsible for providing camera gating and gain modulation. The Delay Unit delays the laser pulse synchronization signal for precise gating timing. It has a delay range of 0-50 ns with a time resolution of 10 ps. It can be triggered by the laser trigger (provided by the laser module) with frequencies ranging from 20 to 80 MHz. It is also responsible for triggering the HRI delay unit and consequently the image intensifier (TTL signal). The delay and the trigger parameters of this device are controlled via software.

A microscope module is placed between the objective (Achroplan 40x/NA=0.45, Zeiss, Wetzlar, Germany) and the PicoStar HR camera. A high-pass yellow filter with a cutting wavelength of 500 nm is used to prevent excitation light from reaching the camera photocathode. Figure 4 shows the schematic of the prototype instrumentation

The fluorescence lifetime image is built from the image volume containing a series of images of decreasing intensity, acquired for each gate delay. The decay profile and parameters for a given pixel of the fluorescence lifetime image are obtained by fitting the intensity values across equivalent pixels of the image volume, using non-linear regression.

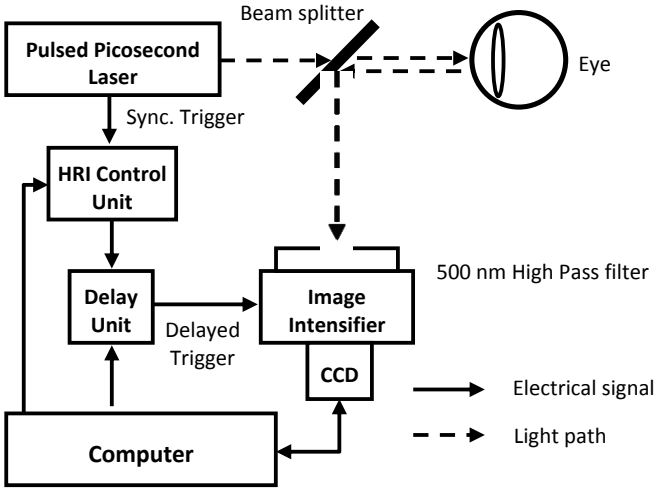

Figure 4 – Corneal fluorescence microscope for *in vivo* FAD metabolic imaging: prototype schematic.

3.2. Results

So far, we only performed test and characterization measurements on this setup. Lateral resolution and field of view were measured in reflectance mode (i.e. without the high pass filter at the camera input), using an USAF 1951 test target, yielding 4.3 µm and 159 x 119 µm, respectively.

The microscope accuracy was verified using solutions of standard fluorophores with single-exponential decays [18] both in the nanosecond and sub-nanosecond ranges, representative of fluorescence lifetime ranges associated with the components of FAD double decay. Results are presented in Table 2.

Table 2. Fluorescence lifetime measurements on standard fluorophores

Fluorophore	Gate Width (ps)	Measured lifetime (ps)	Reference Lifetime (ps)
Erithrosin B in methanol	300	485.3	480 [18]
	400	487.2	
Coumarin 153 in methanol	300	4169.6	4180 [18]
	400	4241.7	
	500	4171.3	
	600	4116.9	
	700	4146.3	

The results are in very good agreement with the reference values. For long lifetimes, the effect of the gate width is negligible. The sample standard deviation for all Coumarin 153 values is 46.2 ps, a value that corresponds to an excellent precision, despite the gate width variation: for a level of confidence of 95%, the repeatability is 90.5 ps, a value that represents 2.2% of the reference lifetime. The accuracy error is 10.8 ps, less than 0.3% of the reference lifetime.

For shorter lifetimes, comparable to the gate widths, the accuracy decreases and the system overestimates the fluorescence lifetime. The accuracy error was 1.3% of the reference lifetime of Erithrosin B. This is still a very good accuracy.

4. Conclusions

Ex-vivo measurements with rat corneas show that it is possible to image FAD fluorescence from corneal epithelial layer and to retrieve information from its fluorescence decay parameters that can be correlated to metabolic activity. A direct relation between corneal cell metabolism and corneal FLIM remains to be demonstrated and requires controlled metabolic alterations, induced by electron transport chain inhibitors, like cyanide.

The prototype of a time-gated fluorescence lifetime microscope for *in vivo* FAD imaging of corneal tissues has the precision and the accuracy, as well as the timing and lateral spatial resolutions, required for such measurements. Adequate axial resolution for optical sectioning of different corneal layers will be addressed on a second version of the microscope system with the implementation of structured illumination technique [19]. Although the system is based on an intensified camera with single-photon sensitivity, it remains to be proved that an adequate signal-to-noise ratio is achieved with an acquisition time compatible to the requirements of in vivo examination of ocular tissues.

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